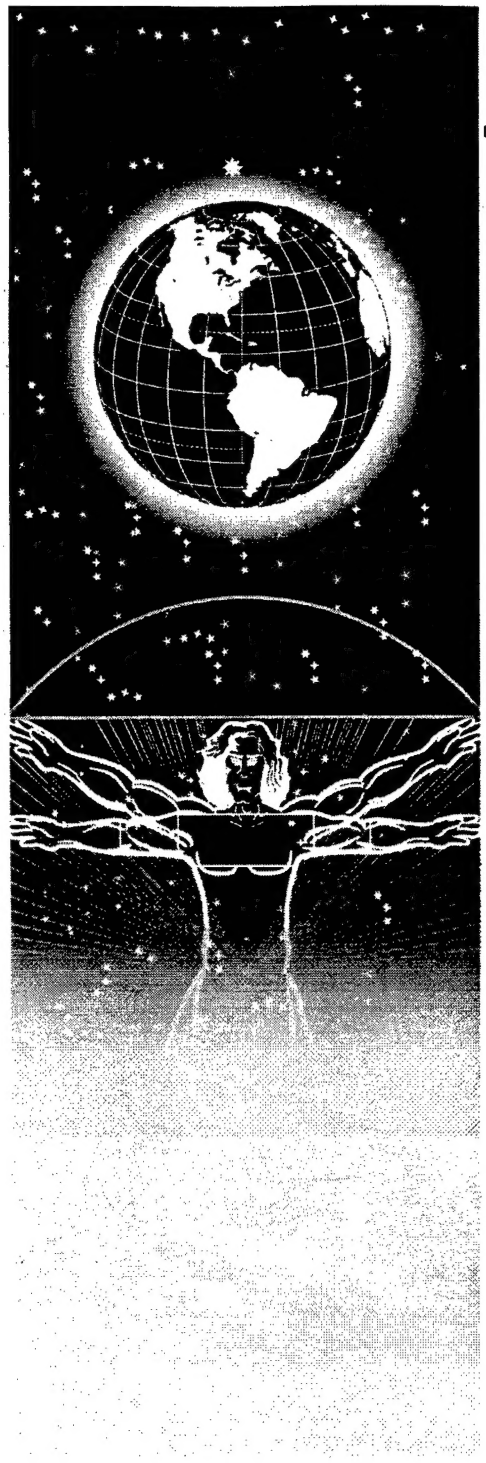
AL/OE-TR-1996-0162



UNITED STATES AIR FORCE ARMSTRONG LABORATORY

Developmental Toxicity Screen of Ammonium Perchlorate Using *Hydra attenuata*

P.D. Confer

GEO-CENTERS, INC.
7 WELLS AVENUE
NEWTON, MA 02159

R.E. Wolfe
E.R. Kinkead

MANTECH ENVIRONMENTAL, INC.
P.O. BOX 31009
DAYTON, OH 45437-0009

December 1996

19970515 072

Approved for public release;

distribution is unlimited.

Occupational and Environmental Health Directorate
Toxicology Division
2856 G St.
Wright-Patterson AFB OH 45433-7400

DTIC QUALITY INSPECTED 1

NOTICES

When US Government drawings, specifications or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Armstrong Laboratory. Additional copies may be purchased from:

NATIONAL TECHNICAL INFORMATION SERVICE
5285 PORT ROYAL ROAD
SPRINGFIELD, VIRGINIA 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

DEFENSE TECHNICAL INFORMATION CENTER
8725 JOHN J. KINGMAN RD STE 0944
FT BELVOIR VA 22060-6218

DISCLAIMER

This Technical Report is published as received and has not been edited by the Technical Editing Staff of the Armstrong Laboratory.

TECHNICAL REVIEW AND APPROVAL

AL/OE-TR-1996-0162

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

TERRY A. CHILDRESS, Lt Col, USAF, BSC
Director, Toxicology Division
Armstrong Laboratory

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1996	Final - June 1995 - October 1996

4. TITLE AND SUBTITLE
Developmental Toxicity Screen of Ammonium Perchlorate Using *Hydra Attenuata*

5. FUNDING NUMBERS
Contract F41624-96-C-9010
PE 62202F
PR 7757
TA 7757A1
WU 7757A112

6. AUTHOR(S)
P.D. Confer, R.E. Wolfe, and E.R. Kinkead

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
ManTech - Geo-Centers Joint Venture
P.O. Box 31009
Dayton, OH 45437-0009

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Armstrong Laboratory, Occupational and Environmental Health Directorate
Toxicology Division, Human Systems Center
Air Force Materiel Command
Wright-Patterson AFB OH 45433-7400

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
AL/OE-TR-1996-0162

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Ammonium perchlorate (AP) is an oxidizer used as a component in solid rocket propellants, munitions, and fireworks. An *in vitro* developmental toxicity screen was performed to determine the developmental hazard index (A/D ratio) for AP using the hydra assay. *Hydra attenuata* have the capability for whole body regeneration. The assay employs exposing both adult hydra and "artificial embryos" composed of disassociated hydra cells to test compounds to investigate developmental toxicity. Results from the hydra assay of AP indicated the minimal effective concentration required to elicit a toxic response tin the adult hydra and in the regenerating hydra were 600 mg A:/L and 350 mg AP/L, respectively. The A/D ratio of 1.71 determined that AP should not be considered a primary developmental toxin in the context of this assay.

14. SUBJECT TERMS
Developmental Toxicity *In Vitro* Screen *Hydra Attenuata* Ammonium Perchlorate

15. NUMBER OF PAGES
38

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

THIS PAGE INTENTIONALLY LEFT BLANK

TABLE OF CONTENTS

LIST OF TABLES

PREFACE

This is one of a series of technical reports describing results of the experimental laboratory programs conducted at Armstrong Laboratory, Toxicology Division (AL/OET), Wright-Patterson Air Force Base, Ohio by the ManTech/Geo-Centers Joint Venture contract. This document serves as a final report on the hydra assay for developmental toxicity of ammonium perchlorate. The research described in this report began in June 1995 and was completed in October 1996 under Department of the Air Force Contract Numbers F33615-90-C-0532 and F41624-96-C-9010. Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, AL/OET. Darol E. Dodd, Ph.D., served as Program Manager for the ManTech/Geo-Centers Joint Venture contract.

This work was initiated under the direction of Daniel J. Caldwell, Ph.D. The authors gratefully acknowledge Dr. E. Marshall Johnson, Dr. Lois D. Newman, and Mr. Navid Haghdoost for hydra assay training, the initial supply of *Hydra attenuata*, and for their guidance during the hydra laboratory preparation. The authors also gratefully acknowledge the technical assistance of David L. Courson, SrA Frank M. Dessauer, David H. Ellis, Marcia L. Feldmann, Richard J. Godfrey, Willie J. Malcomb, Jerry W. Nicholson, and William B. Sonntag. The support of Four Seasons Environmental, Inc. in maintaining the hydra laboratory's heating and air-conditioning systems was also greatly appreciated.

ABBREVIATIONS

A/D ratio	Adult toxic (A) to developmentally toxic (D) ratio
AP	Ammonium perchlorate
C	Centigrade
$CaCl_2$	Calcium chloride
cm	Centimeter
DoD	United States Department of Defense
EDTA	Ethylenediaminetetraacetic acid
Encl	Hydra farm enclosure
EPA	United States Environmental Protection Agency
g	Gram
h	Hour(s)
KCl	Potassium chloride
L	Liter
MAC	Minimal affective concentration
mOhm-cm	MegaOhms per centimeter
$MgSO_4$	Magnesium sulfate anhydrous
min	Minute(s)
mg	Milligram
mL	Milliliter
mm	Millimeter
mM	Millimolar
N	Normal
NaOH	Sodium hydroxide
NCEA	US EPA National Center for Environmental Assessment
RfD	Reference Dose
SD	Standard deviation
TES	{2[Tris(hydroxymethyl)methylamine}-1-ethanesulfonic acid}
µL	Microliter
×g	Times gravity

SECTION I

INTRODUCTION

Ammonium perchlorate (AP) is a class 1.1 oxidizer that is used as a component in solid rocket propellants, munitions and fireworks (CPIA, 1989; TERA, 1996). When AP is used as an oxidizer in solid propellants, the resulting exhaust contains hydrogen chloride gas which has possible long range acid rain implications (Nieder et al., 1990). AP is listed by the Environmental Protection Agency (EPA) as a class B2 carcinogen, which means that it is classified as a probable human carcinogen based on the following criteria: inadequate evidence from epidemiologic studies (or no data) and sufficient evidence from animals (SJRA, 1994). The toxicity of AP is relevant to the Department of Defense (DoD) and the National Aeronautics and Space Administration since the production and storage of AP has resulted in contaminated soils and groundwaters. Although replacement propellants, such as ammonium dinitramide, are being considered, the United States government is still responsible for the costly remediation of these AP-contaminated sites.

In 1992, a provisional reference dose (RfD) of 1×10^{-4} mg/kg/day for AP was developed by the National Center for Environmental Assessment, formerly the Environmental Criteria and Assessment Office (Dollarhide, 1992). This provisional RfD was based on a study by Stanbury and Wyngaarden (1952) in which potassium perchlorate was administered to humans with the

autoimmune disorder known as Graves' Disease. In 1995, based on studies by Stanbury and Wyngaarden (1952), Shigan (1963) and Männistö et al. -(1979), the EPA recommended that RfDs in the range of 1 to 5E-4 mg/kg-day were appropriate (NCEA, 1995; TERA, 1996). The provisional RfD for perchlorate is currently of concern to the DoD and a consortium of government and industry scientists known as the Perchlorate Study Group. There are minimal data on AP and restricted data exist for potassium perchlorate in the literature (Dollarhide, 1992). An abundance of data exists on perchlorate's effects on humans who suffer from Graves' Disease; however there is limited data on chronic exposures in normal humans and animals at concentrations likely found in environmental exposures (TERA, 1996). The only dose-response data that exists estimates the threshold dose of AP based on hormonal data from AP-treated Sprague-Dawley rats (Caldwell, 1995; King, 1995). Rats are considered to be more sensitive to fluctuations in thyroid hormones than humans (Hill et al., 1989; Capen, 1992). There currently are no data available on the reproductive or developmental toxicity of ammonium perchlorate for animals or humans.

The Hydra Assay is performed using the fresh water coelenterate *Hydra attenuata* as the test species. *Hydra attenuata* is the most primitive invertebrate composed of complex tissues and organs, and it is the highest form that has the capability for whole body regeneration. The assay employs the use of both adult hydra and "artificial embryos," composed of disassociated hydra cells, to investigate the potential toxicity of test compounds. Artificial embryos (also called pellets) are

prepared by disassociating adult hydra into their component cells (Gierer et al., 1972). These cells can be reaggregated randomly into the artificial embryo (Johnson, 1980) which will regenerate into adult hydra within a few days under normal conditions. In order for the pellets to regenerate into new adult hydra, the "embryo" must accomplish most, if not all, of the developmental events required during true embryogenesis (Johnson, 1990).

Test chemicals may affect one or more of these developmental events, causing abnormal development and/or death of the artificial embryo. The concentration of the test chemical which causes abnormal development in the embryo may or may not cause an effect in the adult hydra. For this reason, both adult hydra and the artificial embryos are tested concurrently. The lowest concentration of test chemical that causes death in the intact adult hydra is compared to the lowest concentration that produces death in the developing artificial embryo. The adult toxic (A) to developmentally toxic (D) ratio (A/D ratio) is calculated using these concentrations.

The A/D ratio, also referred to as the developmental hazard index, is predictive of a chemical's hazard potential in standard laboratory animals and man (Johnson and Gabel, 1982). A low A/D ratio (<3) predicts a test chemical being toxic to an embryo only at levels which will also cause toxic signs in the adult animal. A high A/D ratio (≥ 3) reveals a chemical's teratogenic hazard, displaying a toxic effect in the developing embryo while causing little to no toxicity in the adult (Johnson et al., 1988).

This study was performed to obtain an A/D ratio for ammonium perchlorate to determine its potential developmental toxicity through use of the hydra assay developmental toxicity screen.

SECTION II

MATERIALS AND METHODS

Test Material

Ammonium Perchlorate (AP)

Source/Supplier:	Aldrich Chemical Company, Milwaukee, Wisconsin
CAS No.:	7790-98-9
Purity:	99.8%
Density:	1.4 g/mL at 20 °C

Because AP is listed as a Department of Transportation oxidizer, only limited quantities of AP were stored and the U.S. Air Force, Armstrong Laboratory, Toxicology Division assumed the responsibility of retaining an archive sample.

Test Species

Hydra attenuata used in this study were produced in the hydra laboratory at Armstrong Laboratory, Toxicology Division, Wright-Patterson Air Force Base, Ohio. *Hydra attenuata* are the only hydroids with which a successful hydra assay may be performed. Polyps of the species *Hydra attenuata* live in fresh water, they are not complicated by algae associations, and they derive all nutrition by feeding (Johnson et al., 1988).

The hydra colony was produced and housed in shallow Plexiglas aquaria which provided controlled aeration (referred to as farms). The hydra farms and test hydra were maintained within a Plexiglas enclosure. The temperature within the laboratory and the enclosure was maintained near 18 ± 2 °C using an attached cooling system. This temperature allows for optimal growth of the hydra (Johnson et al., 1992). Temperatures of ≥ 24 °C or ≤ 12 °C adversely affect normal growth of hydra. Hydra were maintained in a water-based medium (hydra medium) which contained 1.0 mM $CaCl_2$ dihydrate, 0.458 mM TES (sodium salt), and 0.012 mM EDTA. Ultra-purified water (18 mOhm-cm) was used in the preparation of all solutions used throughout the study. The pH of the medium was adjusted to 6.90-6.95 using 0.5 N NaOH. The hydra were fed regularly and allowed to propagate naturally by budding. *Artemia nauplii* (brine shrimp), hatched in 1% saline solution, were provided as food for the hydra. Bowls of hatching brine shrimp were started on a daily rotation to provide a continuously fresh food supply for the hydra. These bowls were maintained in the laboratory under controlled temperature.

Prior to testing, hydra were group housed within the hydra farms. The group-housed hydra were fed three days per week, more often if the numbers of hatched shrimp allowed. The hydra farm was cleaned on a regular schedule to deter any bacterial or algal growth.

The test hydra were fed daily for 3 days prior to testing, but were not fed while on study. Test hydra were maintained in groups of approximately 4000 (4 mL of adult hydra) in separate

aquaria (4 in. deep by 8 in. wide) containing hydra medium during the 3 days prior to testing. During exposure test hydra were in 35 mm × 12 mm glass hydra test dishes (three adult hydra or pellets per test dish).

Experimental Design

The preparation of regenerating artificial hydra embryos and the performance of the Hydra Assays were accomplished following the methods in "The Hydra Assay Manual," Johnson et al., 1992.

The hydra assay for AP consisted of three consecutive experiments, each experiment required testing of both adult hydra and regenerating artificial hydra embryos (pellets). The pH of test Solution A was adjusted (using approximately 0.1 mL of 0.5 N NaOH) to within the pH range of 6.90 to 6.95 for all experiments. Experiment 1 established the concentrations of test material required to produce the toxic endpoint (death). Experiment 1 was a range-finding study to designate the appropriate concentrations for Experiments 2 and 3, which determine the minimal affective concentration (MAC). Experiment 2 utilized a more narrow range of concentrations of test material for more precise resolution of the MAC. Experiment 3 was a repeat of Experiment 2 for confirmation of the MAC. If the MACs from Experiments 2 and 3 were not in close agreement (within the same or adjacent concentration test dishes), the assay would have been repeated.

Control dishes were filled with 4 mL assay reaggregate media (pellets) or assay hydra medium (adults). Test dishes contained the appropriate medium and ascending concentrations of test material so that the total volume used in any test dish was 4 mL (4000 µL). The volume of any solution added to the hydra test dish did not exceed 10% (400 µL) of the total volume of the test dish because the hydra would not survive due to the medium being too dilute. The test material solutions were added directly to dishes already containing the appropriate volume of reaggregate or hydra medium. Doses were selected following the procedures described in "The Hydra Assay Manual" (Johnson et al., 1992). At the 4, 18, 26, 42, and 66 h observations, new test and control test dishes were prepared, and the adults and pellets were transferred into the new solutions after each observation was recorded.

Assay test dishes were kept covered on the countertop during the period from time zero (0 h) through the 4-h observations. Assay test dishes were also covered from 4 h through 90 h, but were housed within the hydra farm enclosure during non-observation periods. Temperatures were recorded for the laboratory and the hydra farm enclosure for each observation time point.

Assay of Developmental Hydra

Artificial hydra embryos (pellets) were produced from disassociated cells of adult hydra. Pellet preparation began by placing 2.0 mL adult hydra into a clean 15-mL conical glass centrifuge tube containing 3.5 mL reaggregate media (3.9 mM KCl,

6.6 mM $CaCl_2$ dihydrate, 0.63 mM $MgSO_4$, 6.6 mM sodium citrate, 6.6 mM sodium pyruvate, 12.0 mM TES buffer). Phenol red was also included in the media as a pH indicator. The pH of the reaggregate media was adjusted to 6.90-6.95 with 0.5 N NaOH prior to use in the assay. Amikacin sulfate USP (Elkins-Sinn, Inc., Cherry Hill, NJ) was also added to the reaggregate media (2 µL/mL media) to inhibit microbial growth. After incubating in the reaggregate media for 30 min, the adult hydra were disassociated by repeated shearing (disassociation) with a glass fire-polished pipette. The resulting suspension was left undisturbed for 6 min and then the supernatant was collected. This procedure was repeated two to three times depending upon the amount of intact hydra left in the centrifuge tube. The pooled supernatant from the disassociations was centrifuged at 300×g for 5 min and then decanted, leaving a volume of fluid approximately equal to the volume of the cell pellet. The cells were resuspended in the tube by gentle mixing, and the resulting slurry was drawn up into 3-cm pieces of polyethylene tubing. The pieces of tubing containing cells were placed into 2.5 mL microcentrifuge tubes and centrifuged at 200×g for 5 min. The pellets formed by the centrifugation consisted of randomly aggregated cells. These "pellets" or artificial hydra embryos were ejected into test dishes containing reaggregate media with or without test material. The time the last pellet was placed into a test dish was 0 h for the developmental portion of the hydra assay experiment.

Three or more pellets were placed into each test dish. Pellets were observed visually at 0 h to ensure viability.

Pellets were observed using a dissecting microscope and observations were recorded 4, 18, 26, 42, 66, and 90 h after the pellets were placed into the test dishes. Observations consisted of recording the stage of development for each pellet. At the 4-h observations, three viable pellets were allowed to remain in each test dish and all other pellets were discarded. These three pellets continued on study for each experiment. Normally developing pellets are solid at 4 h. Pellets appear "hollow" at 18 h of development. At 26 h, normal pellets have tentacle buds, which appear randomly across the pellet as small bumps. At 42 h, normal pellets' tentacle buds begin to elongate into true tentacles. By 66 h, hypostomes begin to appear near the tentacles. Hypostomes are the openings in the top portion of the body of hydra through which they consume food. Ninety hours after beginning to develop, the pellets consist of hydra polyps. The area under the hypostomes will have elongated into the body of a hydra polyp. If allowed to continue to grow, the polyps will separate into individual adult hydra.

The toxic endpoint for the pellet was death. Once any two pellets in one test dish were dead, the concentration in that dish was considered an affective dose. If viable pellets remained within a test dish in which a pellet was found dead, the dead pellet was removed and the surviving pellets allowed to continue through the experiment. Once the 90-h observation was made, the experiment was complete. The MAC was then recorded for the artificial embryos.

Assay of Adult Hydra

Hydra assay medium was prepared by adding Amikacin (2 µL/mL) to hydra medium to prevent bacterial growth. The pH was then adjusted to 6.90-6.95 using 0.5 N NaOH. Three normal adult hydra were placed into each test dish containing either 4 mL of hydra assay medium (control) or assay medium containing the appropriate volume of test material. Adult hydra were visually observed at 0 h to ensure viability. Adult hydra observations were performed using a dissecting microscope. Microscopic observations were recorded 4, 18, 26, 42, 66, and 90 h after the hydra were placed into the test dishes. Following the 90-h observation, the experiment was concluded.

Adult hydra react to a change in their environment (i.e., test material) by contracting their tentacles. Sometimes contraction of the body also occurs. This reaction is called "clubbing." Adult hydra undergoing stress will continue to contract their tentacles until they are very short. The contraction continues under stress until the tentacles are barely discernible, and the hydra physically appear to look like tulip flowers. Hydra will not survive once reaching the "tulip" stage. They will eventually disintegrate, which is recorded as death. The toxic endpoint for adult hydra was death or the "tulip stage." When any two hydra in a test dish died or reached the tulip stage, the concentration in that test dish was considered an affective dose. The MAC was recorded for the adult hydra in each experiment.

Test Material Stock Solutions

Stock Solution A was prepared by adding 200 mg (0.200 g) of AP to 20 mL hydra medium. The pH of Stock Solution A was adjusted (by addition of 0.1 mL 0.5 N NaOH) to a pH of 6.93. Further dilution of Stock Solution A was made to create the other stock solutions.

1 mL Stock A to 9 mL hydra media = Stock B
1 mL Stock B to 9 mL hydra media = Stock C
1 mL Stock C to 9 mL hydra media = Stock D
1 mL Stock D to 9 mL hydra media = Stock E
1 mL Stock E to 9 mL hydra media = Stock F

Determination Of A/D Ratio

The A/D ratio for the test material was calculated using the average MACs from Experiments 2 and 3 using the following equation:

MAC adult hydra ÷ MAC artificial embryo = A/D ratio.

Statistics

Standard statistical analyses are not applicable for results from the hydra assay due to the small number of invertebrates used per concentration. Hydra respond to environmental conditions in a stereotypical manner. The assay's endpoints take into account any differences by specifying that an effect equals the "observed effect" in two out of three invertebrates per test concentration. Since experiments were performed using a wide

range of test material concentrations, and the experiment which determined the affective concentration was repeated, a clear and obvious concentration-response relationship is evident for the assay. The endpoints for the hydra assay are specific and conclusive for both the adult and the developing artificial embryo, and statistical analysis would be unreasonable (Newman et al., 1990).

SECTION III

RESULTS

The observations recorded during each of the three experiments for the Hydra Assay of AP are listed in Tables 1 through 6. Timepoints in which a test dish of adult hydra or pellets reached the toxic endpoint are in bold type on each table. Temperatures of the hydra laboratory and the hydra farm enclosure (Encl) at each of the observation timepoints are included on these tables. The average temperature (± SD) in the laboratory was 19.4 ± 0.6 °C, and the average temperature (± SD) in the enclosure was 18.6 ± 0.2 °C. All experiments were performed using hydra media as the diluent for AP. Hydra adults and pellets were exposed to concentrations of AP from 1000 through 0.001 mg AP/L, and to media only (control).

Adult hydra displayed normal dose-response reactions during Experiment 1 (Table 1). The 1000 mg AP/L adult hydra were all dead by the 66-h observation. At 90 h, all adult hydra remained viable in the 100 mg AP/L through 0.001 mg AP/L and in the control test dishes. The minimal affective concentration (MAC) for the adult hydra was 1000mg AP/L.

Pellets in the 1000 mg AP/L test dish were all dead by the 18-h observation (Table 2). All pellets in the remaining test dishes were hollow at this time. Surviving pellets displayed normal advancing developmental stages through 90 h. The MAC for

the developmental portion of Experiment 1 was determined to 1000 mg AP/L.

Since both the adult and developing pellets were observed to have a MAC at 1000 mg AP/L, this concentration was chosen as the highest dosing level for both forms of hydra for the final experiments (Experiments 2 and 3) that would determine the A/D ratio for AP. For these experiments, test dishes with concentrations of AP from 100 through 1000 mg AP/L, and a control, were used. All adult hydra survived through 26-h treatment during Experiment 2 (Table 3). By 66 h, 2 adult hydra per test dish had reached the toxic endpoint (death or tulip stage) in the 1000, 900, 800, 700, and 600 mg AP/L test dishes. All adult hydra which had not reached the toxic endpoint by 66 h remained viable throughout the remainder of the study, except one hydra in the 400 mg AP/L which reached the tulip stage by the 90-h observation. The adult MAC for Experiment 2 was 600 mg AP/L.

All pellets in Experiment 2 survived through the 4-h observation (Table 4). By 18 h, all pellets were dead in the 1000 and 900 mg AP/L test dishes. By 26 h, 2 or 3 pellets per test dish were dead in the 800, 700, 600, and 500 mg AP/L test dishes. At 42 h, 2 pellets in the 400 mg AP/L were dead. One pellet per test dish died in the 300 and 200 mg AP/L test dishes by 66 and 90 h, respectively. All other pellets continued to develop through 90 h. The developmental MAC was 400 mg AP/L for Experiment 2.

Experiment 3 was a repetition of Experiment 2. This procedure was done to assure that the MACs for both adult and developing hydra obtained in Experiment 2 were accurate. As in Experiment 2, at least 2 adult hydra per test dish reached the toxic endpoint (death or tulip stage) in the 1000, 900, and 800 mg AP/L test dishes by the 66-h observation (Table 5). By 90-h, 2 adult hydra per test dish had reached the toxic endpoint in both the 700 and 600 mg AP/L test dishes. All remaining adult hydra were viable though the 90-h observation. Control hydra remained normal throughout Experiment 3. The adult MAC for Experiment 3 was 600 mg AP/L.

The developmental observations of Experiment 3 repeated similarly to the observations recorded during Experiment 2. The only differences noted were that the toxic endpoint for pellets in the 600 and 500 mg AP/L test dishes occurred at 42 h instead of 26 h; and by 66 h, 2 pellets were dead in the 300 mg AP/L test dish (Table 6). The MAC for the developmental portion of Experiment 3 was 300 mg AP/L.

A/D Ratio

The A/D ratio determined by the hydra assay of ammonium perchlorate was **1.71** (adult MAC of 600 mg AP/L divided by the average developmental MAC of 350 mg AP/L).

Table 1. Experiment Number 1 Adult Hydra Observations
MAC 1000 mg AP/L

Test Dish Concentration	4 hours	18 hours	26 hours	42 hours	66 hours	90 hours
1000 mg AP/L	2 clubbed severe, 1 clubbed slight	3 short	1 short severe, 2 short	1 dead, 2 short	2 dead	
100 mg AP/L	2 clubbed slight, 1 normal	2 clubbed, 1 clubbed slight	2 clubbed, 1 clubbed slight	2 clubbed, 1 clubbed slight	3 clubbed	2 clubbed, 1 clubbed slight
10 mg AP/L	1 clubbed, 1 clubbed slight, 1 normal	3 clubbed slight	3 clubbed slight	3 clubbed slight	3 clubbed slight	3 clubbed slight
1.0 mg AP/L	2 clubbed slight, 1 normal	2 clubbed slight, 1 normal	2 clubbed slight, 1 normal	2 clubbed slight, 1 normal	2 clubbed slight, 1 normal	1 clubbed, 2 clubbed slight
0.1 mg AP/L	1 clubbed, 1 clubbed slight, 1 normal	3 clubbed slight	1 clubbed, 2 clubbed slight	1 clubbed, 2 clubbed slight	1 clubbed, 2 clubbed slight	1 clubbed, 2 clubbed slight
0.01 mg AP/L	1 clubbed, 1 clubbed slight, 1 normal	3 clubbed slight	1 clubbed, 2 clubbed slight	1 clubbed, 2 clubbed slight	1 clubbed, 1 clubbed slight, 1 normal	2 clubbed, 1 clubbed slight
0.001 mg AP/L	1 clubbed slight, 2 normal	3 normal	3 normal	3 normal	3 normal	3 clubbed slight
CONTROL 0.0 mg AP/L	3 normal	3 normal	3 normal	3 normal	3 normal	2 clubbed slight, 1 normal
Temp °C	Lab 19.1	Lab 20.2 Encl 18.4	Lab 19.4 Encl 18.6	Lab 19.4 Encl 18.4	Lab 20.0 Encl 18.5	Lab 20.0 Encl 18.6

Table 2. Experiment Number 1 Pellet Observations
MAC 1000 mg AP/L

Test Dish Concentration	4 hours	18 hours	26 hours	42 hours	66 hours	90 hours
1000 mg AP/L	3 solid	3 dead				
100 mg AP/L	3 solid	3 hollow	3 tentacle buds slight	3 elongated	2 hypostomes, 1 elongated slight,	2 polyp slight, 1 hypostome
10 mg AP/L	3 solid	3 hollow	2 tentacle buds, 1 tentacle bud slight	2 elongated slight, 1 tentacle bud	2 hypostomes, 1 elongated	3 polyps slight
1.0 mg AP/L	3 solid	3 hollow	2 tentacle buds, 1 tentacle bud slight	2 elongated, 1 elongated slight	2 hypostomes, 1 hypostome slight	3 polyps slight
0.1 mg AP/L	3 solid	3 hollow	3 tentacle buds	2 elongated, 1 elongated slight	2 hypostomes, 1 elongated	1 polyp, 1 polyp slight, 1 hypostome
0.01 mg AP/L	3 solid	3 hollow	3 tentacle buds	3 elongated	2 hypostomes, 1 hypostome slight	3 polyps slight
0.001 mg AP/L	3 solid	3 hollow	3 tentacle buds	3 elongated	2 hypostomes, 1 hypostome slight	1 polyp, 2 polyps slight
CONTROL 0.0 mg AP/L	3 solid	3 hollow	3 tentacle buds	3 elongated	3 hypostomes	3 polyps
Temp °C	Lab 19.1	Lab 20.2 Encl 18.4	Lab 19.4 Encl 18.6	Lab 19.4 Encl 18.4	Lab 20.0 Encl 18.5	Lab 20.0 Encl 18.6

Table 3. Experiment Number 2 Adult Hydra Observations
MAC 600 mg AP/L

Test Dish Concentration	4 hours	18 hours	26 hours	42 hours	66 hours	90 hours
1000 mg AP/L	3 clubbed	3 short	3 short severe	1 dead, 2 short severe	**2 dead**	
900 mg AP/L	3 clubbed	3 short	3 short severe	1 dead, 2 short severe	**2 dead**	
800 mg AP/L	3 clubbed	2 short, 1 clubbed severe	2 short severe, 1 short	1 dead, 2 short severe	**2 dead**	
700 mg AP/L	2 clubbed, 1 clubbed slight	3 clubbed severe	1 short, 2 clubbed severe	3 short severe	**2 dead**, 1 short severe	1 short severe
600 mg AP/L	3 clubbed slight	2 clubbed, 1 clubbed slight	2 clubbed severe, 1 clubbed	2 short severe, 1 short	**2 tulip**, 1 short	2 dead, 1 short
500 mg AP/L	3 normal	3 clubbed slight	3 clubbed	2 short severe, 1 short	1 tulip, 2 short	1 dead, 2 short
400 mg AP/L	3 clubbed slight	2 clubbed, 1 clubbed slight	1 short, 2 clubbed	1 short severe, 2 short	3 short severe	1 tulip, 2 short severe
300 mg AP/L	2 clubbed slight, 1 normal	2 clubbed slight, 1 normal	2 clubbed, 1 clubbed slight	2 clubbed, 1 clubbed slight	1 clubbed severe, 2 clubbed	1 clubbed severe, 2 clubbed
200 mg AP/L	3 normal	3 normal	3 clubbed slight	3 clubbed slight	3 clubbed	2 clubbed, 1 clubbed slight
100 mg AP/L	3 normal	3 normal	3 clubbed slight	3 clubbed slight	2 clubbed slight, 1 normal	2 clubbed slight, 1 normal
CONTROL 0.0 mg AP/L	1 clubbed slight, 2 normal	3 normal	3 normal	3 normal	3 normal	3 clubbed slight
Temp °C	Lab 18.7	Lab 19.8 Encl 18.9	Lab 19.5 Encl 19.3	Lab 19.8 Encl 18.4	Lab 19.5 Encl 18.5	Lab 19.6 Encl 18.6

Table 4. Experiment Number 2 Pellet Observations
MAC 400 mg AP/L

Test Dish Concentration	4 hours	18 hours	26 hours	42 hours	66 hours	90 hours
1000 mg AP/L	3 solid	**3 dead**				
900 mg AP/L	3 solid	**3 dead**				
800 mg AP/L	3 solid	1 dead, 2 solid	**2 dead**			
700 mg AP/L	3 solid	3 solid	**3 dead**			
600 mg AP/L	3 solid	2 hollow slight, 1 solid	**3 dead**			
500 mg AP/L	3 solid	3 hollow slight	**2 dead,** 1 semi-solid	1 dead		
400 mg AP/L	3 solid	3 hollow slight	3 tentacle buds	**2 dead,** 1 elongated	1 dead	
300 mg AP/L	3 solid	3 hollow slight	3 tentacle buds	3 elongated	1 dead, 2 tentacle buds	2 tentacle buds
200 mg AP/L	3 solid	3 hollow	3 tentacle buds	2 elongated, 1 elongated slight	2 elongated, 1 elongated slight	1 dead, 2 hypostomes slight
100 mg AP/L	3 solid	3 hollow	3 tentacle buds	3 elongated	1 hypostome, 2 hypostomes slight	3 polyps
CONTROL 0.0 mg AP/L	3 solid	3 hollow	3 tentacle buds	3 elongated	3 hypostomes	3 polyps
Temp °C	Lab 18.7	Lab 19.8 Encl 18.9	Lab 19.5 Encl 19.3	Lab 19.8 Encl 18.4	Lab 19.5 Encl 18.5	Lab 19.6 Encl 18.6

Table 5. Experiment Number 3 Adult Hydra Observations
MAC 600 mg AP/L

Test Dish Concentration	4 hours	18 hours	26 hours	42 hours	66 hours	90 hours
1000 mg AP/L	3 clubbed	3 clubbed	1 short, 2 short severe	1 dead, 2 short severe	**2 dead**	
900 mg AP/L	3 clubbed	3 clubbed	3 short	3 short severe	**1 dead,** **1 tulip,** 1 short severe	1 dead, 1 tulip
800 mg AP/L	3 clubbed	1 clubbed severe, 2 clubbed	3 short	3 short severe	**1 dead,** **2 tulip**	2 dead
700 mg AP/L	3 clubbed slight	3 clubbed	1 short, 2 clubbed	3 short severe	1 tulip, 2 short severe	**1 dead,** **1 tulip,** 1 short severe
600 mg AP/L	1 clubbed, 2 clubbed slight	3 clubbed	3 clubbed	2 short severe, 1 short	1 tulip, 1 short severe, 1 short	**2 tulip,** 1 short
500 mg AP/L	2 clubbed slight, 1 normal	2 clubbed slight, 1 normal	2 clubbed, 1 clubbed slight	2 short, 1 clubbed	3 short	3 short
400 mg AP/L	1 clubbed slight, 2 normal	1 clubbed slight, 2 normal	3 clubbed slight	3 clubbed	3 clubbed	3 clubbed
300 mg AP/L	3 normal	3 normal	1 clubbed, 1 clubbed slight, 1 normal	1 clubbed, 1 clubbed slight, 1 normal	1 clubbed, 2 clubbed slight	3 clubbed
200 mg AP/L	3 normal	3 normal	3 normal	1 clubbed slight, 2 normal	2 clubbed slight, 1 normal	1 clubbed, 2 clubbed slight
100 mg AP/L	3 normal	3 normal	3 normal	3 normal	3 normal	1 clubbed slight, 2 normal
CONTROL 0.0 mg AP/L	3 normal	3 normal	3 normal	3 normal	3 normal	3 normal
Temp °C	Lab 18.7	Lab 20.2 Encl 19.0	Lab 18.6 Encl 18.6	Lab 18.6 Encl 18.6	Lab 18.5 Encl 18.6	Lab 18.8 Encl 18.7

Table 6. Experiment Number 3 Pellet Observations
MAC 300 mg AP/L

Test Dish Concentration	4 hours	18 hours	26 hours	42 hours	66 hours	90 hours
1000 mg AP/L	3 solid	**2 dead,** 1 semi-solid	1 dead			
900 mg AP/L	3 solid	**2 dead,** 1 solid	1 dead			
800 mg AP/L	3 solid	1 dead, 2 solid	**2 dead**			
700 mg AP/L	3 solid	3 solid	**3 dead**			
600 mg AP/L	3 solid	1 hollow slight, 2 solid	1 dead, 2 solid	**2 dead**		
500 mg AP/L	3 solid	3 hollow slight	1 dead, 2 tentacle buds slight	**2 dead**		
400 mg AP/L	3 solid	3 hollow slight	3 tentacle buds	**3 dead**		
300 mg AP/L	3 solid	2 hollow, 1 hollow slight	3 tentacle buds	1 dead, 1 elongated, 1 solid	**2 dead**	
200 mg AP/L	3 solid	3 hollow	3 tentacle buds	2 elongated, 1 elongated slight	2 elongated, 1 elongated slight	2 hypostomes slight, 1 elongated
100 mg AP/L	3 solid	3 hollow	3 tentacle buds	2 elongated, 1 elongated slight	2 hypostomes slight, 1 elongated	2 polyps, 1 hypostome
CONTROL 0.0 mg AP/L	3 solid	3 hollow	3 tentacle buds	2 elongated, 1 elongated slight	2 hypostomes slight, 1 elongated	3 polyps
Temp °C	Lab 18.7	Lab 20.2 Encl 19.0	Lab 18.6 Encl 18.6	Lab 18.6 Encl 18.6	Lab 18.5 Encl 18.6	Lab 18.8 Encl 18.7

SECTION IV

DISCUSSION

A/D ratios calculated from hydra assays have been compared to the A/D ratios from standard Segment II-type studies (Johnson et al., 1988). A comparison was made of A/D ratios obtained for 61 chemicals in which both hydra assays and Segment II-type studies were performed. Of the 61 chemicals, 57 of them had A/D ratios for hydra and Segment II-type studies which were in agreement. The hydra assay is 90% accurate and has a low incidence of false negatives (Johnson et al., 1988).

The developmental hazard index, or A/D ratio, of 1.71 for AP would predict that AP would only be toxic to a developing embryo at levels which would also cause maternal toxicity. Although no data are available on human developmental toxicity of AP, Crooks and Wayne (1960) reported twelve infants of mothers who were treated with 600 to 1000 mg perchlorate/day for Graves' Disease during gestation were born with no abnormalities (with the exception of one infant born with a very slightly enlarged thyroid which returned to normal size in 6 weeks). Under the conditions of the hydra assay performed in this laboratory, AP should not be considered a primary developmental toxin.

SECTION V

REFERENCES

Caldwell, D.J., E.R. Kinkead, L. Narayanan, J.H. King, Jr., and D.R. Mattie. 1995. Results of a Fourteen Day Oral-dosing Toxicity Study of Ammonium Perchlorate. JANNAF Propulsion and Subcommittee Joint Meeting, Tampa, FL. December 5, 1995.

Capen, C.C. 1992. Pathophysiology of Chemical Injury of the Thyroid Gland. *Toxicology Letters* **64/65**:381-388.

Chemical Propulsion Information Agency (CPIA). 1989. Critical Technology. Johns Hopkins University Applied Physics Laboratory, Laurel, MD. November 1989.

Crooks, J. and E.J. Wayne. 1960. A Comparison of Potassium Perchlorate, Methylthioracil, and Carbimazole in the Treatment of Thyrotoxicosis. *Lancet* **1**:401-404.

Dollarhide, J.S. 1992. Risk Assessment Issue Paper for: Provisional Non-cancer and Cancer Toxicity Values for Potassium Perchlorate. US EPA Environmental Criteria and Assessment Office, Cincinnati, OH. December 2, 1992.

Gierer, A., S. Berking, H. Bode, C.N. David, K. Flick, G. Hansmann, H. Schaller, and E. Trenkner. 1972. Regeneration of hydra from reaggregated cells. *Nature* **239**:98-101.

Hill, R.N., L.S. Erdreich, O.E. Paynter, P.A. Roberts, S.L. Rosenthal, and C.F. Wilkinson. 1989. Thyroid Follicular Cell Carcinogenisis. *Fundamental and Applied Toxicology* **12**:629-697.

Johnson, E.M. 1980. A subvertebrate system for rapid determination of potential teratogenic hazards. *J. Environ. Pathol. Toxicol.* **4**:153-156.

Johnson, E.M. and B.E.G. Gabel. 1982. Application of the hydra assay for rapid detection of developmental hazards. *Journal of the American College of Toxicology* **1**(3):57-71.

Johnson, E.M., L.M. Newman, B.E.G. Gabel, T.F. Boerner, and L.A. Dansky. 1988. An analysis of the hydra assay's applicability and reliability as a developmental toxicity prescreen. *Journal of the American College of Toxicology* **7**(2):111-126.

Johnson, E.M. 1990. Practical Application of Systems for Rapid Detection of Potential Teratogenic Hazards. In: *Advances in Modern Environmental Toxicology.* Vol. 3, Assessment of Reproductive and Teratogenic Hazards. Princeton Scientific Publishing Co., Inc., Princeton, NJ. pp. 77-91.

Johnson, E.M., B.E.G. Gabel, L.M. Newman, and R. Giacobbe. 1992. The Hydra Assay Manual. Thomas Jefferson Medical College, Philadelphia, PA.

King, J.H. 1995. Effects of Ammonium Perchlorate on the Thyroid Hormone Levels of the Sprague-Dawley Rat. Thesis AFIT/GEE/ENV/95D-09 Presented to the Air Force Institute of Technology, Wright-Patterson AFB, OH.

Männistö, P.T., T. Ranta, and J. Leppäluoto. 1979. Effects of Methylmercaptoimidazole (MMI), Propylthioracil (PTU), Potassium Perchlorate ($KCLO_4$) and Potassium Iodide (KI) on the Serum Concentrations of Thyrotrophin (TSH) and Thyroid Hormones in the Rat. *Acta Endocrinologica* **91**(1979):271-281.

National Center for Environmental Assessment (NCEA). 1995. Review of Proposed RfD for Perchlorate. US EPA NCEA, Cincinnati, OH.

Nieder, E.G., G.T. Bowman, and P.L. Stefanski. 1990. Clean Propellant Feasibility Motor Demonstration. FO4611-89-C-0028. Atlantic Research Corporation, Gainesville, VA.

Newman, L.M., R.L. Giacobbe, L-J. Fu, and E.M. Johnson. 1990. Developmental Toxicity Evaluation of Several Cosmetic Ingredients in the Hydra Assay. *Journal of the American College of Toxicology* **9**(3):361-365.

Science and Judgment in Risk Assessment (SJRA). 1994. National Research Council. U.S. EPA Contract CR818293-01-0. Washington: National Academic Press.

Shigan, S.A. 1963. Substantiation of the Maximum Permissible Concentration of Ammonium Perchlorate in Water of Reservoirs. Translated from the original Russian by the National Air Intelligence Center, September 26, 1994 (NAIC-ID (RS) T-0624-94). *Gigiena i sanit.* **28**(8):8-14.

Stanbury, J.B. and J.B. Wyngaarden. 1952. Effect of Perchlorate on the Human Thyroid Gland. *Metabolism.* **1**:533-539.

Toxicology Excellence for Risk Assessment (TERA). 1996. Proposed Perchlorate Reference Dose (RfD). Prepared for the Perchlorate Study Group, September 20, 1996.